Effect of calcium on the hydration of casein. I. Water vapour sorption and fine structure of calcium caseinates compared with sodium caseinates in the pH range 4·6–8·0

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Summary. Hydration of Ca and Na caseinates which were prepared from whole casein at different pH levels (range 4·7–8·0) was determined by means of water vapour sorption measurements in the water activity (a_w) range 0·58–0·95. Water uptake of Ca caseinates was systematically lower than that of Na caseinates prepared at equal pH, the differences increasing with increasing pH and a_w. Plots of the water content *v.* the amount of added hydroxide at constant a_w revealed a linear relationship between water uptake and cation content, suggesting that the increase in water uptake is mainly determined by the amount and type of cations associated with the caseins. In the a_w range tested, Ca^{2+} adsorbed about 2–7 and Na^+ 3–18 mol water/mol of cation. This implies that replacement of one mol of casein-bound Na^+ by Ca^{2+} is accompanied by a displacement of 1–11 mol water at a_w 0·58–0·95. The loss of water is a consequence of conformational changes induced by the chelating and cross-linking effects of Ca^{2+}, which also lead to micelle formation.

The interaction between Ca and caseins plays a key role in the formation and structure of casein micelles. It also influences the behaviour of the caseins during the manufacture of milk products. The most striking effects of the binding of the divalent ion to whole casein, or its principal components, α_{s1}- and β-casein, are the aggregation and the decrease in solvation. Isolated κ-casein, which comprises about 14 % of whole bovine casein, differs somewhat in its behaviour. It remains soluble even at high concentrations of Ca^{2+}. In the presence of the divalent ion, κ-casein forms micelles with either α_{s1}- or β-casein, stabilizing the latter against precipitation. Ca-induced aggregation and precipitation of caseins have been investigated in great detail using a variety of methods. However, little information is available on Ca-induced changes in hydration. A decrease in the amount of solvation water has been observed when Ca^{2+} was added to purified caseins (Waugh *et al.* 1971; Slattery & Waugh, 1973) or casein micelle suspensions in dilute solutions (Green & Marshall, 1979). Also, a negative correlation has been found between the Ca content of casein micelles and their solvation (Sood *et al.* 1979*a*, *b*).

Ca-binding by caseins is pH-dependent (Carr, 1953; Zittle *et al.* 1958; Dickson & Perkins, 1971). A pH increase is generally accompanied by a greater Ca-binding capacity. Also, a pH shift is observed upon Ca-binding by caseins, due to the displacement of protons (Kiermeier, 1952; Kiermeier & Galanos, 1957; Waugh *et al.* 1971). The solvent systems used to study the influence of pH on Ca-binding and hydration usually contained both Ca^{2+} and monovalent cations, mainly Na^+.

Therefore, the changes in hydration observed were to some extent caused by the transformation of Na into Ca caseinate. A study of the water-binding properties of the pure caseinates is an essential preliminary to a better understanding of the way in which Ca^{2+} interacts with casein and influences its hydration. Furthermore, it seems worthwhile to make this type of study not only in dilute solutions but also in semi-dry systems with reduced water activity (a_w). Various milk products in which the interaction of Ca with casein is important have low a_w levels compared with those of most model systems used in the past.

The present paper is concerned with hydration of pure Ca and Na caseinates in the a_w range 0·58–0·95. Water vapour sorption was measured as a function of pH and water activity, using isopiestic techniques. In addition, the fine structure of the caseinates was studied by transmission electron microscopy.

MATERIALS AND METHODS

Materials

Whole casein was prepared from milk of Simmental cows by acid precipitation. With vigorous stirring diluted skim-milk was slowly brought to pH 4·6 using 5% acetic acid. The precipitate was washed 3 times with deionized water and redissolved in water at pH 7·0 with the aid of 2 M-NaOH. After removing undissolved particles by filtration the precipitation procedure was repeated twice and the final preparation lyophilized. Electrophoretic examination on polyacrylamide gel (Groves & Kiddy, 1968) revealed a mean composition of 47% a_s-, 38% β-, 15% κ-, and 1·4% γ-casein. The acid casein contained 75 μmol Ca, 13 μmol Mg, 500 μmol Na, 41 μmol K and 25 μmol P/100 g dry protein. The cations were analysed by atomic absorption spectrophotometry after mineralization in hot 65% nitric acid. Phosphorus content was measured using a standard photometric method (Schweizerisches Lebensmittelbuch, 1969).

To obtain Ca and Na caseinates at various pH levels, 5 g portions of acid casein were dispersed in 50 ml water and 0·01 M-$Ca(OH)_2$ or 0·1 M-NaOH were added. The suspensions were allowed to equilibrate over 3 d and then lyophilized. The pH range tested (pH 4·7 to about 8·0) corresponded to a cation content of 0·07–45 mmol Ca or 0·50–84 mmol Na/100 g dry casein.

Methods

Water sorption measurements. Water vapour sorption isotherms at 25·0 ± 0·1 °C were determined using an isopiestic method. The apparatus and technique of this method are described in detail by Gál (1975) and Gál & Hunziker (1977). The following saturated salt solutions were used to obtain equilibrium water contents in the water activity range 0·58–0·95: NaBr ($a_w = 0{\cdot}576 \pm 0{\cdot}004$), KI ($0{\cdot}689 \pm 0{\cdot}002$), NaCl ($0{\cdot}753 \pm 0{\cdot}001$), KCl ($0{\cdot}843 \pm 0{\cdot}003$), $CaCl_2$ (0·902), and KNO_3 ($0{\cdot}936 \pm 0{\cdot}006$) (Stokes, 1949; Greenspan, 1977). The highest humidity level ($0{\cdot}950 \pm 0{\cdot}001$) was produced with a sulphuric acid solution (Rüegg, 1980). In preliminary measurements, the dry samples were exposed directly to each humidity (integral sorption). This procedure was found not to be suitable because of extremely long equilibration times and irregular weight gain curves. Better results were achieved by increasing the humidity stepwise from the lowest to the highest level at 7-d intervals (differential sorption). The values of equilibrium water content are based on the dry weight obtained after heating for 6 h at 60 °C in a vacuum oven and subsequent cooling over P_2O_5.

Mathematical analysis of sorption data. The sorption equation according to the model of Guggenheim, Anderson and de Boer, (G.A.B. equation), proposed for food materials by van den Berg (1981), has been used to fit an isotherm to the experimental points. This 3-parameter equation can be rearranged into a second degree polynomial and is then mathematically identical to the isotherm equation derived from the model of Hailwood & Horrobin (1946):

$$\frac{a_w}{W} = \alpha \,.\, a_w^2 + \beta \,.\, a_w + \gamma$$

(W: water uptake on dry basis; α, β, γ: coefficients related to number of primary sorption sites, heat of sorption and factor correcting properties of water in multilayers). Regression analyses were made using BMDP computer programs (BMDP Statistical Software 1981, University of California Press, Berkeley, CA, USA) and special plots were drawn with the aid of DISSPLA subroutines (DISSPLA Version 8·2, Integrated Software Systems Corp., San Diego, CA, USA.)

Electron microscopy. The caseinates were fixed with glutaraldehyde and acrolein and embedded in agar for better handling during the dehydration and epoxy resin embedding procedure (Blanc *et al.* 1980). The very fine agar fibrils are visible in the electron microscope but can clearly be distinguished from casein aggregates (Fig. 1). The procedure of Goldsmith (1967) was used to estimate the distribution of casein particles from the distribution of their cross-sections observed in the electron microscope. (A slice thickness of 30 nm was assumed).

RESULTS AND DISCUSSION

When $Ca(OH)_2$ was added to the suspension of whole casein a colloidal dispersion of spherical particles was formed above a pH of about 5·8. The Ca caseinate particles had a volume/surface average diam. of d_{vs} = 380 nm. (The observed number-average diam. was 200 nm.) The distribution width, expressed as coefficient of variation of d_{vs}, was 54 %. Fig. 1 shows electron micrographs typical for the undissolved Ca caseinate in the pH region before and after formation of a colloidal dispersion. The material in the pH range 4·6 to ~ 5·7 appears mostly homogeneous. The spherical particles showed a substructure, but not as pronounced as natural and artificial micelles which also contain phosphate and citrate (Schmidt *et al.* 1974; Knoop *et al.* 1979).

During the course of titration with NaOH the casein gradually dissolved. Compared with the individual casein components (Bingham, 1971) the solubilization of whole casein occurred at higher pH values. The solubility increased from about 0·05 mg/ml at pH 5·7 to 2·0 mg/ml around pH 6·0.

At the same pH levels, the Na caseinates always absorbed a higher amount of water than in Ca caseinates. Fig. 2 shows 2 water sorption isotherms typical for the caseinates in the a_w range 0·55–0·95. The difference between the water uptake of Na and Ca caseinate increased with increasing pH and a_w. This can be clearly seen in Fig. 3, where the measured sets of isotherms are represented in 3-dimensional plots. The surface formed by the isotherms of Ca caseinates if flat over the whole range tested compared with that for Na caseinates.

The curves parallel to the pH axis in Fig. 3 represent isopsychric lines. They are not smooth because they were drawn by connecting points, representing equal a_w-levels, on the regression lines of the isotherms. Two isopsychric functions, one for a medium and the other for a high water activity, are shown in detail in Fig. 4. In

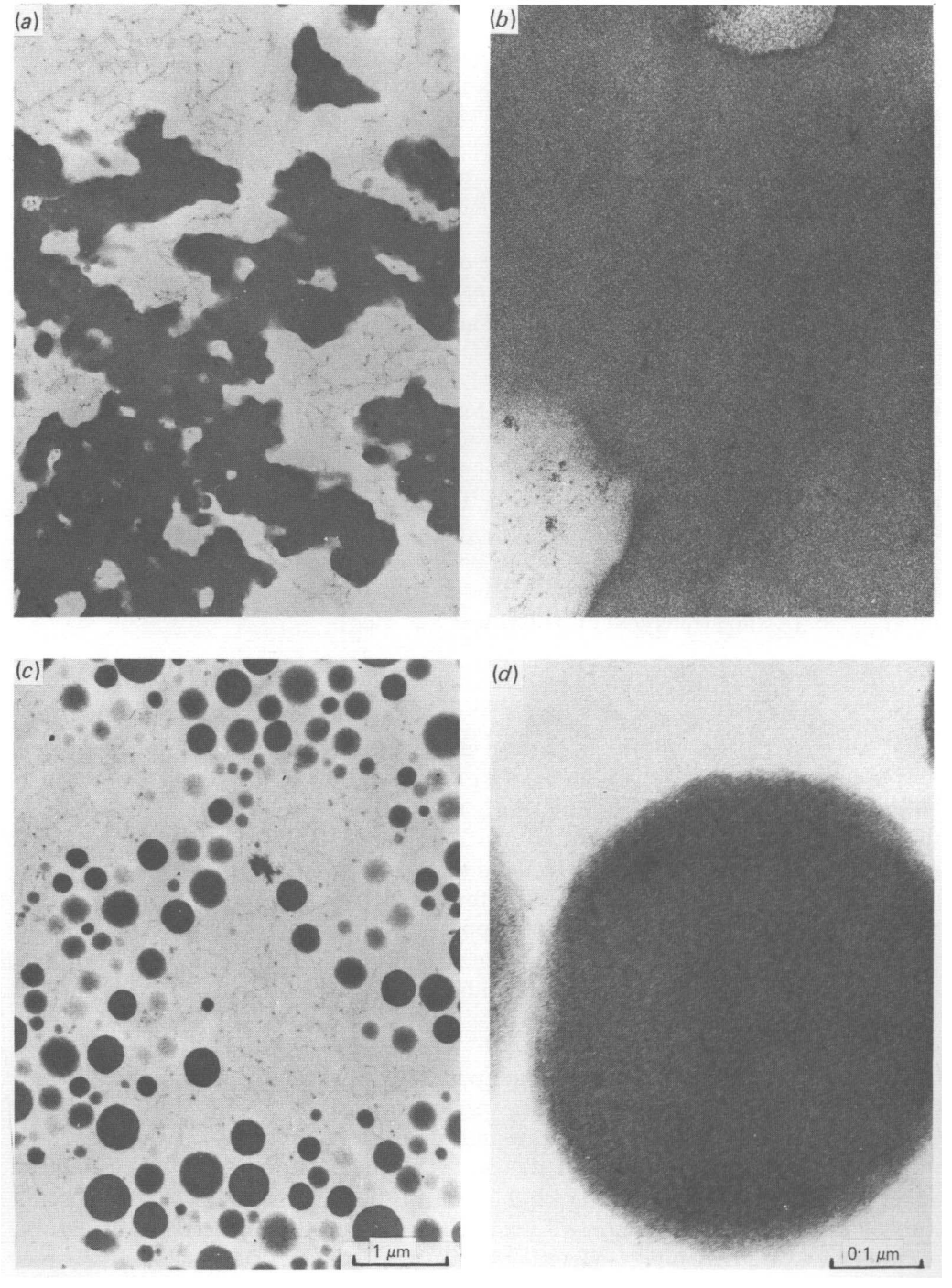

Fig. 1. Electron micrographs of Ca caseinates. (*a*) and (*b*) Fine structure of insoluble Ca caseinate at pH 5·1 (0·033 mmol Ca/g casein), typical for the samples in the pH range of 4·6 to 5·8 (0·002 to ~ 0·18 mmol Ca/g casein). (*c*) and (*d*) Micelles of Ca caseinate at pH 6·1 (0·24 mmol Ca/g casein), typical for the dispersions formed in the approximate pH range 5·8–8·0 (0·18–0·45 mmol Ca/g casein).

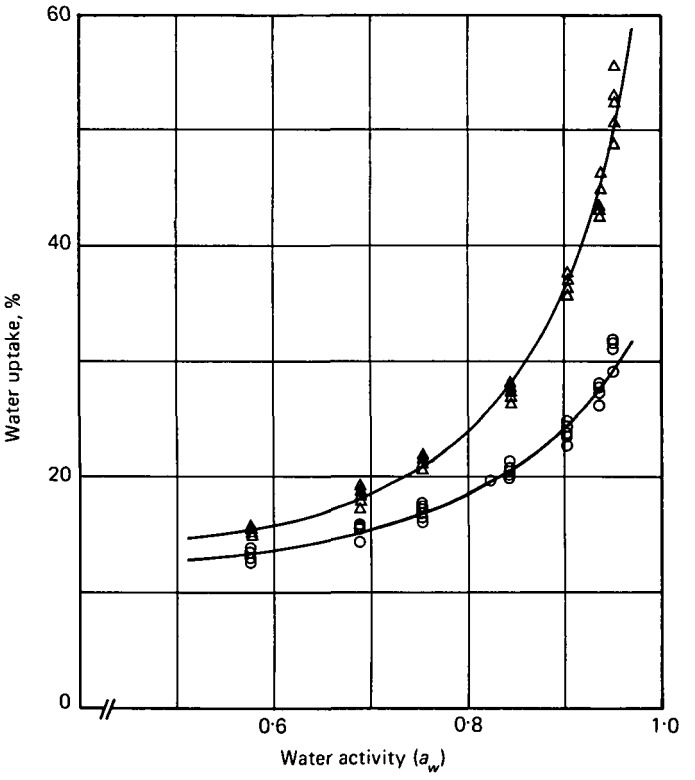

Fig. 2. Water sorption isotherm of Ca and Na caseinate at 25 °C and pH 7·5. The curves correspond to the G.A.B.-isotherm obtained by regression analysis. △, Na caseinate; ○, Ca caseinate.

the pH range 4·6 to about 5·5, the water uptake by the 2 caseinates was not significantly different. At higher pH levels, however, the isopsychric curves begin to separate. The separation coincides with the formation of Ca caseinate micelles.

In Figs 2 and 3 the comparison of water absorption by caseinates is made in relation to pH. It must be considered that in the case of Ca and Na caseinate an equal degree of protonization represents a difference in cation content of a factor of about 2. Therefore, it is meaningful to discuss the water sorption capacity of the caseinates also as a function of the amount of cation associated with the proteins. The data in Fig. 4(*a*) have been replotted in Fig. 4(*b*) as a function of the concentration of Ca and Na in units of mmol/g dry and cation-free casein. The experimental points now apparently follow a straight line. This phenomenon has already been observed for Na caseinates and casein hydrochlorides in a limited a_w and pH range (Signer & Gál, 1961; Rüegg & Blanc, 1976). Linear least squares regression lines are drawn in Fig. 4(*b*). Although close inspection of the residuals of the regression analysis indicated a sigmoid shaped isopsychric curve, the deviation from linearity was not statistically significant. The slope and intercept values for the regression lines are given in Table 1. The intercept corresponds to the water content of whole casein at pH 4·7, before the addition of hydroxide. The slope values represent the contribution of the protein-bound cations. The apparently linear relationship suggests that the increase in water uptake is mainly determined by the amount and type of cations bound by whole casein. The amount of water associated with the peptide chain seems to play a minor role. The slope values thus are an estimate of the hydration of the casein-bound cations in units of g water/100 mmol of Ca^{2+} or Na^{+}.

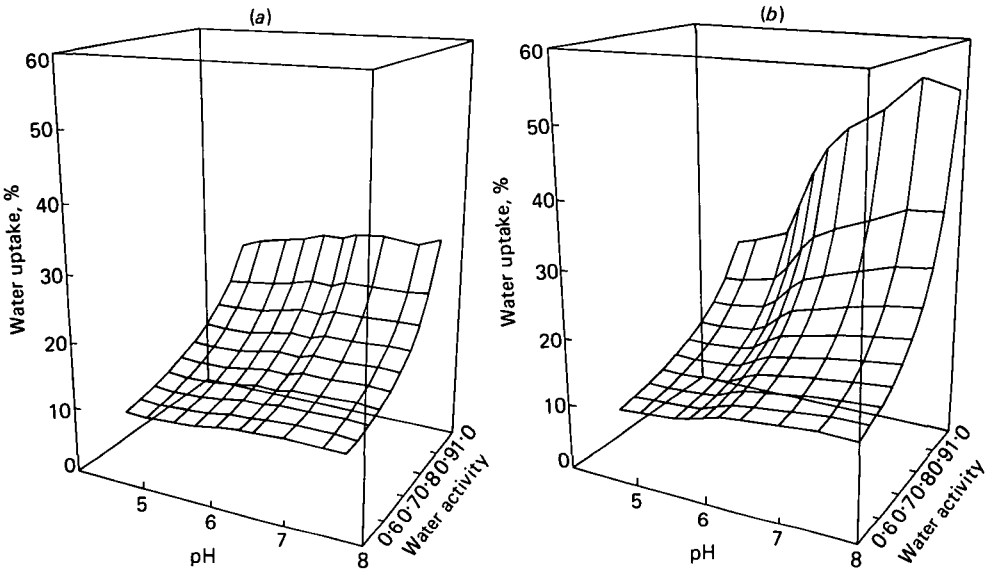

Fig. 3. Water uptake of (*a*) Ca and (*b*) Na caseinates in the high a_w-range as a function of pH at 25 °C. The 2 sets of sorption isotherms (water content *v.* a_w functions) were obtained by regression analysis of the experimental points and represent G.A.B.-functions (details are given in the experimental section). Water content *v.* pH curves in the surface are isopsychric lines.

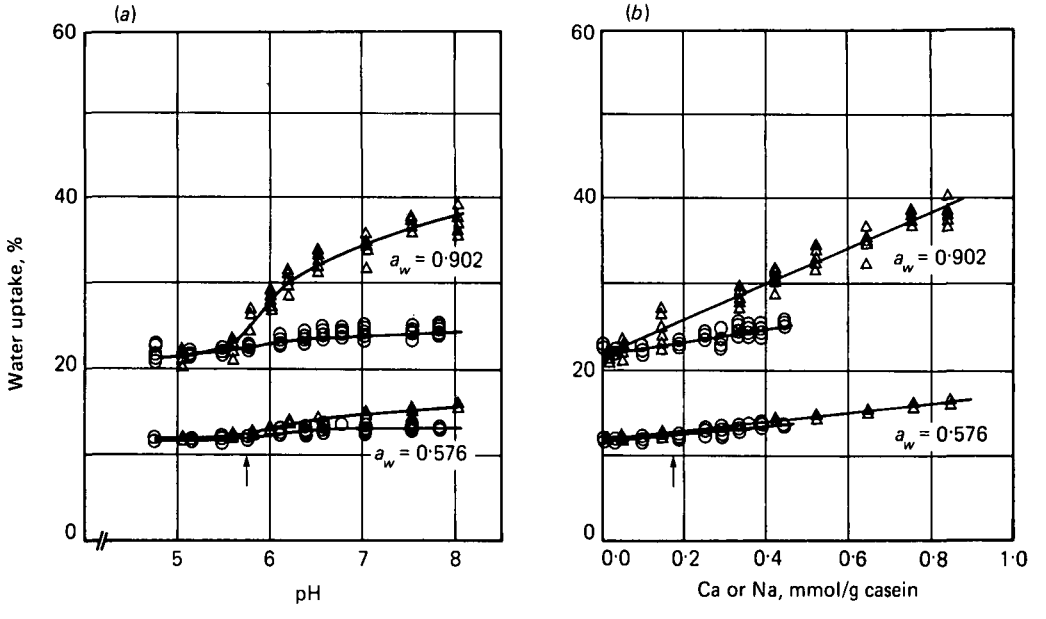

Fig. 4. Isopsychric curves for Ca and Na caseinates at 25 °C. (*a*) Water uptake at a_w = 0·576 and 0·902 as a function of pH. (*b*) Same data plotted as a function of the amount of $Ca(OH)_2$ and NaOH added to isoelectric whole casein. Linear least squares regression lines are shown for the data in Fig. 4(*b*). Arrows point to the region of formation of Ca caseinate micelles. (Ordinate: water content on dry and metal-free basis). △, Na caseinate; ○, Ca caseinate.

Table 1. *Isopsychric lines for Ca and Na caseinates. Regression coefficients of the apparent linear relationship between water uptake (W) at 25 °C and molar concentration of cations (c): $W = \alpha + \beta . c$**

	Ca caseinate					Na caseinate				
a_w	α	s_α	β	s_β	R^2	α	s_α	β	s_β	R^2
0·576	11·59	0·10	4·60	0·34	0·705	11·81	0·04	5·11	0·09	0·979
0·689	13·46	0·10	4·87	0·36	0·714	13·47	0·10	6·95	0·22	0·943
0·753	15·09	0·13	5·12	0·43	0·639	15·21	0·07	8·57	0·15	0·980
0·843	18·22	0·11	6·44	0·36	0·801	18·26	0·11	12·90	0·24	0·979
0·902	21·58	0·14	7·45	0·48	0·751	21·68	0·23	20·43	0·50	0·964
0·936	23·99	0·19	10·17	0·67	0·745	24·46	0·37	26·27	0·84	0·939
0·950	26·65	0·28	12·52	1·00	0·685	28·14	0·48	32·40	1·03	0·944

* Water uptake (W) in g/100 g dry and cation-free protein; cation concentration in mmol/g protein.
s_α, s_β, standard deviations.
R^2, coefficient of determination.

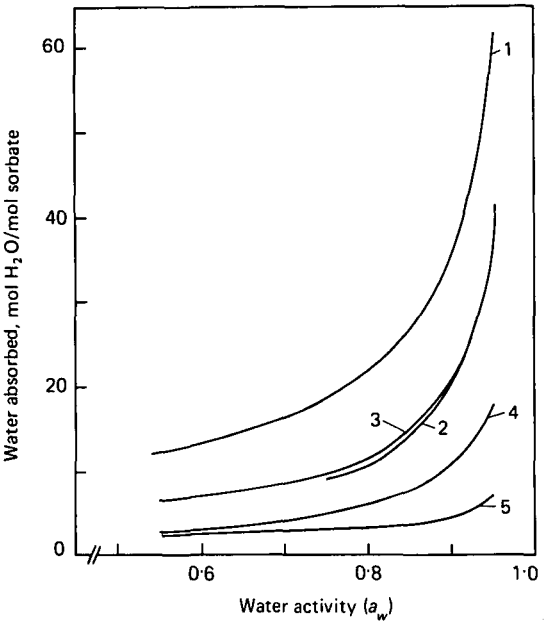

Fig. 5. Hydration of Ca^{2+} and Na^+ in caseinates in the a_w-range 0·58–0·95, compared with the hydration of the corresponding chlorides and NaOH. The sorption isotherms for the individual cations in the caseinates were estimated from the slopes of the isopsychric curves (see Fig. 4(*b*) and Table 1). The isotherms for the chlorides and for NaOH were calculated from data of Robinson & Stokes (1959). 1, $CaCl_2$; 2, NaCl; 3, NaOH; 4, Na^+ (caseinate); 5, Ca^{2+} (caseinate).

It is interesting to note that the hydration of casein-bound Na^+ is systematically higher than that of Ca^{2+}. This can clearly be seen in Fig. 5, which shows the water sorption isotherms of the casein-bound cations. (The slope values in Table 1 have been recalculated on a molar basis and plotted against the water activity). For comparison, water uptake of NaCl, NaOH and $CaCl_2$ has been calculated from other data (Robinson & Stokes, 1959) and the corresponding curves included in Fig. 5. In protein-free aqueous salt solutions, primary hydration numbers for Ca^{2+} are systematically greater than for Na^+. From entropy and ion-mobility data, the number of tightly bound water molecules in a first shell has been estimated as 7–12 for Ca^{2+} and 2–4 for Na^+ (see e.g. review by Kortüm, 1962). Ions of high charge density usually increase

the order of the water structure and have higher primary hydration numbers. The different situation in caseinate systems can be explained by the chelating and cross-linking effect of Ca^{2+}. For steric reasons, water is displaced from the primary and secondary shell of hydration of Ca^{2+} after binding to deprotonized groups at protein side chains. The chelating and cross-linking effects eventually lead to micelle formation. Hydrophobic interactions are also important in the polymerization process as electrostatic repulsion is reduced by Ca^{2+}-binding (Slattery, 1979).

Fig. 5 also shows that the difference between the hydration of Ca^{2+} and Na^{+} in caseinates increases from about 1 to 11 mol water/mol cation in the a_w range 0·70–0·95.

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